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(54) **C-TERMINAL MODIFICATION OF POLYPEPTIDES**

C-TERMINALE MODIFIKATION VON POLYPEPTIDEN

MODIFICATION DE L'EXTREMITE C-TERMINALE DE POLYPEPTIDES

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Description

[0001] The present invention relates to a mutated trypsin comprising an amino acid substitution both at position K60 and D189, and at least one more amino acid substitution by histidine at position N143 or position E151. Such trypsin mutant has a preferred cleavage site comprising the amino acids Xaa_1-Xaa_2-His, wherein Xaa_1 is L, Y or F and Xaa_2 is R or K. The invention also relates to a man-made polypeptide comprising a target peptide and the above cleavage site as well as to a method of producing C-terminally modified target peptides by using this mutated trypsin.

[0002] The use of biologically active peptides, e.g. for pharmaceutical purposes has become more and more important during the past years. Several methods exist to produce such biologically active peptides, for example, the chemical synthesis based on solid phase or solution phase peptide synthesis techniques, or the cultivation of genetically manipulated microorganisms followed by the isolation and purification of the such produced recombinant proteins.

[0003] However, it remains difficult and costly to chemically synthesize polypeptides of more than about 50 amino acids. It also represents a significant task to modify a peptide obtained by chemical peptide synthesis and/or a recombinantly obtained polypeptide at its C-terminal end. One of the most powerful methods to modify polypeptides is through a controlled protein ligation, whereby peptide analogs, unnatural amino acids, stable isotopes, fluorophores, and other biochemically or biophysically important molecules can be specifically incorporated into a polypeptide. One of these methods is based on the introduction - mostly synthetically - of a chemo-selective amino acid, mainly a cysteine which then is modified by a thio-selective reagent attacking the SH-side chain of this amino acid residue. A further alternative is the so-called intein-based protein ligation system, which can generate a protein thioester by proteolysis of a corresponding protein-intein fusion protein (Blaschke, U.K., et al., Methods Enzymol. 328 (2000) 478-496). This method has been successfully applied to introduce unnatural modifications into proteins. However, difficulties remain, e.g., because the target protein must be expressed as a fusion protein together with an intein.

[0004] Recently a few more enzyme-based approaches for peptide ligation and/or C-terminal modification have been described. Breddam and co-workers (e.g. US 5,985,627) describe the use of the serine protease carboxypeptidase Y (CPD-Y) in the C-terminal modification of peptides with fluorescence or affinity labels. This modification is based on the specific ability of CPD-Y to stepwise cleave amino acids off the C-terminal end of polypeptides. This method therefore may be considered to be a specific tool for modification of the C-terminus of a polypeptide. CPD-Y cleaves off the C-terminal amino acid under formation of a peptide-acyl-enzyme-intermediate. This acyl-enzyme-intermediate upon nucleophilic attack is deacylated resulting in a transamidation reaction. The desired transamidation reaction may be accompanied by (un-wanted) side reactions like hydrolysis. It is also possible that more than one C-terminal amino acid is cleaved off, on the other hand also amino acids may be added by this method (Stennicke, H. R., et al., Anal. Biochem. 248 (1997) 141-148 and Buchardt, O., et al., US 5,580,751)

[0005] Abrahmson et al. (e.g. WO 94/18329) described the use of serine protease variants for ligation of peptides. Subtilisin variants are disclosed which have an improved peptide ligase activity. It is, however, necessary for effective peptide ligation to use an appropriate amino terminus protecting group and an appropriate carboxy terminus activating group, respectively, in order to efficiently ligate two peptide substrates.

[0006] Recently sortase-mediated protein ligation has been described as an alternative method in protein engineering (Mao, H., et al., J. Am. Chem. Soc. 126 (2004) 2670-2671). Sortase, an enzyme isolated from *Staphylococcus aureus* catalyses a transpeptidation reaction by cleaving between threonine and glycine in a recognition motif consisting of the amino acids LPXTG and subsequently joining the carboxyl group of threonine to an N-terminal glycine. In nature it catalyses the transpeptidation of the threonine to an amino group of pentaglycine on the cell wall peptidoglycan.

[0007] In the sortase recognition motif LPXTG, X may be the amino acids D, E, A, N, Q, or K. This enzyme has been used to ligate carboxyterminal threonine residues of a peptide or a protein to an N-terminal glycine of a second peptide. As mentioned, sortase requires a recognition motif of five amino acids of which four amino acids (LPXT) will be present within the ligation product.

[0008] Therefore, whereas several methods exist for C-terminal modification of polypeptides there is a tremendous need for alternative or improved methods of C-terminal modification of polypeptides. The inventors of the present invention have found that it is possible to use special trypsin mutants in the C-terminal modification of polypeptides.

[0009] In one embodiment that present invention therefore relates to a mutated trypsin comprising an amino acid substitution both at position K60 and D189, and at least one amino acid substitution by histidine at position N143 or position E151 according to the chymotrypsin nomenclature which corresponds to positions 43, 171, 123, and 131, respectively, of the sequence given in SEQ ID NO:1.

[0010] The skilled artisan is familiar with the so-called chymotrypsin nomenclature as, e.g., described in Hartley, B.S., and Shotton, D.M., The Enzymes, P.D. Boyer (ed.), Vol. 3, (1971), pp. 323-373 and will have no problem in aligning the positions of a variant trypsin with positions given according to the chymotrypsin nomenclature to the corresponding ones of the trypsin sequence of SEQ ID No: 1.

[0011] Position 60 according to chymotrypsin nomenclature corresponds to position 43 of the sequence of mature anionic rat trypsin II from *Rattus norvegicus* as given in SEQ ID NO: 1.

[0012] Position 143 according to chymotrypsin nomenclature corresponds to position 123 of the sequence of mature anionic rat trypsin II from *Rattus norvegicus* as given in SEQ ID NO: 1.

[0013] Position 151 according to chymotrypsin nomenclature corresponds to position 131 of the sequence of mature anionic rat trypsin II from *Rattus norvegicus* as given in SEQ ID NO:1.

[0014] Position 189 according to chymotrypsin nomenclature corresponds to position 171 of the sequence of mature anionic rat trypsin 11 from *Rattus norvegicus* as given in SEQ ID NO: 1.

[0015] Since the skilled artisan is used to express positions referring to the chymotrypsin nomenclature, therefore, in the following the references to specific sequence position, e.g., position K60 or simply position 60 are exclusively based on positions according to the chymotrypsin nomenclature.

[0016] The present invention also relates to the use of a man-made polypeptide comprising a target peptide and a restriction site peptide comprising the cleavage site Xaa_1-Xaa_2-His, wherein Xaa_1 is L, Y or F, and Xaa_2 is R or K, wherein said restriction site peptide overlaps with the target peptide by the amino acid Xaa_1 at the C-terminal end of said target peptide as a substrate of a trypsin mutant as disclosed in the present invention.

[0017] Also provided is a method of producing a C-terminally transacylated target peptide comprising the steps of: (a) providing a polypeptide comprising a target peptide and a restriction site peptide comprising the cleavage site Xaa_1-Xaa_2-His, wherein Xaa_1 is L, Y or F, and Xaa_2 is R or K, wherein said restriction site peptide overlaps with the target peptide by the amino acid Xaa_1 at the C-terminal end of said target peptide, (b) bringing said peptide into contact with a trypsin mutant according to the present invention under conditions allowing for endoproteolytic cleavage after Xaa_1 and formation of an endoprotease target peptide-acyl-intermediate, (c) adding an appropriate nucleophile and (d) upon nucleophilic attack and binding said nucleophile to the C-terminus of the target peptide releasing the mutated trypsin from the endoprotease target peptide-acyl-intermediate.

[0018] In a further embodiment the present invention relates to nucleotide sequences coding for the novel trypsin mutants, to vectors comprising such mutants and to transformed host cells comprising such vectors.

[0019] The mutated trypsin according to the present invention is a trypsin comprising amino acid substitutions at both the positions K60 and D189 and at least one amino acid substitution at position N143 or at position E151. The substitution in position 143 and/or position 151 is by histidine (His).

[0020] Preferably the mutated trypsin according to the present invention comprises either the amino acid E or the amino acid D in position 60, thus replacing the amino acid K normally present in position 60.

[0021] It is also preferred that the mutated trypsin according to the present invention comprises either the amino acid K, the amino acid H, or the amino acid R in position 189, thus replacing amino acid D normally present in that position. A very preferred substitution is with K at position 189.

[0022] In a further preferred embodiment the mutated trypsin according to the present invention comprises mutations in positions 60, 143, 151 and 189. Preferred substitutions in this mutated enzyme are K60E or D, N143H, E151H and D189K or R.

[0023] The above described mutants of trypsin have very interesting and important properties. Such mutants appear to preferentially recognize a binding or cleavage site consisting of 3 amino acids Xaa_1-Xaa_2-His, wherein Xaa_1 is L, Y or F and Xaa_2 is R or K. This restriction site is cleaved by the above mutants after Xaa_1. This is a very important feature, because by using the novel trypsin mutants only one amino acid, i.e. the C-terminal Xaa_1, will remain within the modified target polypeptide.

[0024] To facilitate an understanding of the invention, a brief discussion of the terminology used in connection with the invention will be provided. The present disclosure uses the terminology of Schechter, J., and Berger, A., Biochem. Biophys. Res. Commun. 27 (1967) 157-162, to describe the location of various amino acid residues on the peptide substrate and within the active site of a corresponding proteolytic enzyme.

[0025] According to the terminology proposed by Schechter, J. and Berger, A., supra, the amino acid residues of the peptide substrate are designated by the letter "P". The amino acids of the substrate on the N-terminal side of the peptide bond to be cleaved (the "cleavage site") are designated P_n...P_3, P_2, P_1, with P_n being the amino acid residue furthest from the cleavage site. Amino acid residues of the peptide substrate on the C-terminal side of cleavage site are designated P_1', P_2', P_3'...P_n' with P_n' being the amino acid residue furthest from the cleavage site. Hence, the bond which is to be cleaved (the "cleavage site") is the P_1-P_1' bond.

[0026] The generic formula for the amino acids of the substrate of an endopeptidase (like for example trypsin) is as follows:

P_n-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_n'

[0027] The designation of the substrate binding sites of an endopeptidase is analogous to the designation of amino acid residues of the peptide substrate. However, the binding sub sites of an endopeptidase are designated by the letter "S" and can include more than one amino acid residue. The substrate binding sites for the amino acids on the N-terminal site of the cleavage site are labeled S_n...S_3, S_2, S_1. The substrate binding sub site for the amino acids on the carboxy side of the cleavage site are designated S_1', S_2', S_3', S_n'. Hence, in an endopeptidase, the S_1' sub site interacts with the P_1' group of the peptide substrate and the incoming nucleophile. A generic formula for describing substrate binding

sites of an endopeptidase is:
S_n-S_3-S_2-S_1-S_1'-S_2'-S_3'-S_n'

[0028] The S_1 binding site binds the side chain of the penultimate amino acid, P_1, of the peptide substrate, in case of a trypsin mutant according to this invention the amino acid Xaa_1. The S_1' binding site interacts with the side chain of P_1', in the present case with Xaa_2. Likewise, the S_2' binding site interacts with the side chain of the histidine residue in position P_2'.

[0029] As the skilled artisan will appreciate the present invention may also be carried out with trypsin variants comprising an amino acid substitution both at position K60 and D189, and at least one more amino acid substitution by histidine at position N143 or position E151.

[0030] The term "variant" refers to polypeptides having amino acid sequences that differ to some extent from a native polypeptide sequence. Ordinarily, a variant amino acid sequence will possess at least about 80% homology with the corresponding parent trypsin sequence, and preferably, it will be at least about 90%, more preferably at least about 95% homologous with such corresponding parent trypsin sequence. The amino acid sequence variants possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence of the native amino acid sequence. Preferably sequence homology will be at least 96% or 97%.

[0031] "Homology" is defined as the percentage of residues in the amino acid sequence variant that are identical after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. One such computer program is "Align 2," authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, DC 20559, on December 10, 1991.

[0032] Preferably, a variant of trypsin as disclosed in the present invention in comparison to the corresponding wild-type sequence, comprises 20 amino acid substitutions or less, more preferred 15 amino acid substitutions or less, also preferred 10 amino acid substitutions or less, and also preferred 6 amino acid substitutions or less.

[0033] The modified trypsin of the invention is capable of improved transacylation when compared to the corresponding native trypsin. As used herein, "transacylation" is a reaction in which a peptide fragment C-terminal to the trypsin cleavage site is exchanged for a nucleophile. Transacylation reactions include transthiolation, transesterification and transamidation reactions. "Transamidation" occurs when an amide bond is formed between the nucleophile and the target peptide substrate. In a transamidation reaction, the nucleophile is not necessarily an amino acid. "Transpeptidation" as an important subgroup of transamidation occurs when the nucleophile is an amino acid, or amino acid derivative, such as an amino acid ester or amino acid amide.

[0034] A general transacylation reaction according to the present invention is shown below:

$$\underset{\text{substrate}}{P_n\text{–}P_3\text{–}P_2\text{–}Xaa_1\text{–}Xaa_2\text{–}His\text{–}P_3\text{`}\text{–}P_n\text{`}} + \underset{\text{nucleophile}}{N} \longrightarrow \underset{\text{mutated trypsin}}{}$$

$$\underset{\text{modified substrate}}{P_n\text{–}P_3\text{–}P_2\text{–}Xaa_1\text{–}N} + \underset{\text{cleaved off C-terminus}}{Xaa_2\text{–}His\text{–}P_3\text{`}\text{–}P_n\text{`}}$$

[0035] In the first step, the enzyme attacks the peptide bond between Xaa_1 and Xaa_2, displacing the more C-terminal amino acids and forming a covalent (an acyl) bond between the P_1 residue (Xaa_1 of the target peptide and the enzyme. This intermediate is referred to as a target peptide "peptide-acyl-enzyme intermediate" or briefly as acyl-enzyme intermediate. In the presence of an appropriate nucleophile, under proper conditions, the enzyme causes the nucleophile to add to the cleaved peptide substrate to produce a transacylated product. It is believed that the nucleophile attaches to the carboxyl group of the acyl-enzyme intermediate and displaces the enzyme from the acyl-enzyme intermediate. In this manner, the nucleophile becomes linked to the carboxyl group of the peptide substrate.

[0036] Instead of undergoing a transacylation reaction, the acyl-enzyme intermediate might be deacylated by water to produce a hydrolysis product. The mutated trypsin of the invention is designed to preferentially produce the transacylation product over the hydrolysis product.

[0037] The present invention also relates to a man-made polypeptide comprising a target peptide and a restriction site peptide comprising the cleavage site Xaa_1-Xaa_2-His, wherein Xaa_1 is L, Y or F, and Xaa_2 is R or K, wherein said restriction site peptide overlaps with the target peptide by the amino acid Xaa_1 at the C-terminal end of said target peptide. With other words it relates to a man-made polypeptide comprising a target peptide and a restriction site peptide comprising the cleavage site Xaa_1-Xaa_2-His, wherein Xaa_1 is L, Y or F, and Xaa_2 is R or K, wherein said restriction site peptide and said target peptide share the amino acid Xaa_1 at the C-terminal end of said target peptide. More preferred

Xaa_1 is Y or F, and especially preferred Xaa_1 is Y.

[0038] The term target peptide thus refers to the peptide or polypeptide of the sequence P_n-P_3-P_2- Xaa_1. The target peptide thus includes one amino acid of the cleavage site for the mutated trypsin of this invention, i.e., the amino acid Xaa_1, being either L, Y or F. The term peptide does include polypeptides.

[0039] The term man-made is used to indicate that the peptide sequence is artificial, e.g. it has been designed by a scientist or by a computer. The present invention does not relate to naturally occurring polypeptides comprising the above-defined sequence motif Xaa_1-Xaa_2-His. It merely relates, to man-made, e.g. synthetically or recombinantly produced polypeptides which have been designed to comprise both a target polypeptide as well as a restriction site peptide to comprise the cleavage site Xaa_1-Xaa_2-His with Xaa_1 and Xaa_2 as defined above. According to our definition the target polypeptide has the amino acid Xaa_1 as its C-terminal amino acid. The restriction site peptide comprises at least the amino acids Xaa_2 -His and optionally C-terminal thereto further amino acids. Thus the cleavage site consisting of Xaa_1-Xaa_2-His overlaps with the target polypeptide by one amino acid (Xaa_1) and by two amino acids (Xaa_2-His) with the restriction site peptide. As this skilled artisan will appreciate in principle any polypeptide comprising Xaa_1-Xaa_2-His wherein Xaa_1 and Xaa_2 are as defined above, may be used as a substrate for the trypsin mutants according to the present invention, e.g. in an effort of peptide ligation or in an effort of C-terminal peptide transacylation, e.g. for modification and/or labeling purposes.

[0040] Preferably a target polypeptide according to the present invention consists of 20 to 2000 amino acids. Also preferred is a target peptide consisting of 30 to 1500 amino acids. More preferred such target polypeptide consists of 40-1000 amino acids.

[0041] Preferred target polypeptides are polypeptides used in diagnostic or in therapeutic applications.

[0042] Preferred target polypeptides for example comprise specific binding agents, like antibodies and fragments thereof. Also preferred are specific binding agents obtainable by phage display (see e.g., Allen, J.B., et al., TIBS 20 (1995) 511-516).

[0043] The term antibody refers to a polyclonal antibody, a monoclonal antibody, fragments of such antibodies, as well as to genetic constructs comprising the binding domain of an antibody. Any antibody fragment retaining essentially the same binding properties as the parent antibody can also be used.

[0044] Preferably the target polypeptide comprised in a recombinant polypeptide according to the present invention is a therapeutically active polypeptide. Such therapeutically active polypeptide preferably is selected from the group consisting of a therapeutic antibody, erythropoietin and an interferon. Preferably the therapeutic protein is erythropoietin or an interferon.

[0045] It is obvious to the skilled artisan that only such polypeptides will be used as target polypeptides which do not comprise the sequence motif Xaa_1-Xaa_2-His, with Xaa_1 and Xaa_2 as defined above, as part of their sequence N-terminal to this desired cleavage site. The skilled artisan will have no problems in excluding those polypeptides having a potential cleavage site for a mutated trypsin of the present invention. In the alternative such potential internal cleavage site sequence may be modified by routine mutation and/or cloning techniques to change and/or remove such un-desired internal cleavage site.

[0046] In a preferred embodiment the polypeptide according to the present invention which comprises at or close to its C-terminus the sequence Xaa_1-Xaa_2-His, wherein Xaa_1 and Xaa_2 are as defined above, is produced by recombinant methods. The skilled artisan will have no problem to engineer any desired target polypeptide which is accessible to recombinant production in a way to comprise at or close to the C-terminus the above defined restriction site of Xaa_1-Xaa_2-His.

[0047] The restriction site peptide according to the present invention at least comprises the amino acids Xaa_2 (R or K)-His with Xaa_2 at its N-terminus. It may contain additional amino acids C-terminal thereto which facilitate for example recombinant production or easy purification. In a further preferred embodiment the recombinant polypeptide will comprise as part of the restriction site peptide a so-called His-tag at its C-terminal end which allows for an easy purification by well established chromatographic methods, e.g., use of hexa-His and Ni-NTA-chromatography (Hochuli, E., et al., J. Chromatogr. 411 (1987) 177-184).

[0048] Preferably the above described trypsin mutants are used in a method for C-terminal acylation of a peptide substrate. As the skilled artisan will appreciate, such peptide substrate will comprise a cleavage site for a mutated trypsin according to this invention and the method will comprise the steps of providing an appropriate peptide substrate, bringing said peptide substrate into contact with a trypsin mutant according to the present invention under conditions allowing for endoproteolytic cleavage after Xaa_1 and formation of an endoprotease target peptide-acyl-intermediate, adding an appropriate nucleophile, and upon nucleophilic attack and binding of said nucleophile to the C-terminus of the target peptide releasing the mutated trypsin from the endoprotease target peptide-acyl-intermediate.

[0049] Preferably the above-described mutant trypsins and the above-described polypeptides comprising Xaa_1-Xaa_2-His, with Xaa_1 and Xaa_2, as defined above, are used in a method of C-terminal polypeptide modification by transacylation, e.g. in a method for peptide ligation. In a preferred embodiment the present invention therefore relates to a method of producing a C-terminally transacylated target peptide comprising the steps of: (a) providing a polypeptide

comprising a target peptide and a restriction site peptide comprising the cleavage site Xaa_1-Xaa_2-His, wherein Xaa_1 is L, Y or F, and Xaa_2 is R or K, (b) bringing said peptide into contact with a trypsin mutant according to the present invention under conditions allowing for endoproteolytic cleavage after Xaa_1 and formation of an endoprotease target peptide-acyl-intermediate, (c) adding an appropriate nucleophile and (d) upon nucleophilic attack and binding of said nucleophile to the C-terminus of the target peptide releasing the mutated trypsin from the endoprotease target peptide-acyl-intermediate.

[0050] As used herein, a nucleophile is a molecule that donates a pair of electrons to an electron acceptor, in this case the α-carboxyl carbon of the peptide-acyl-enzyme intermediate, to form a covalent bond.

[0051] Preferably the nucleophile is selected from the group consisting of primary amines, imines, secondary amines, thiol and hydroxyl. Suitable nucleophiles for example include amino acids; amino acid derivatives, such as amino acid esters and amino acid amides; amines, such as ammonia, or benzyl amines.

[0052] The terms "transacylation" or "transacylated" are used to indicate that the C-terminal amino acid of the target peptide (Xaa_1) is bound via covalent bond to the nucleophile. Where the nucleophile is a thiol the transacylation is a thiolation, where the nucleophile comprises a hydroxylic group the transacylation is a esterification and where the nucleophile is an amine the transacylation results in a transamidation. Transamidation reactions are very important and represent a preferred embodiment according to the present invention.

[0053] As the skilled artisan will readily appreciate, appropriate nucleophiles may furthermore comprise modifications which introduce desired properties to the C-terminal end of an appropriate target polypeptide. Preferably the present invention relates to a nucleophile comprising a modification that is selected from the group consisting of a peptide, a peptide amide, a label, a labeled amino acid amide, a labeled peptide, a labeled peptide amide, a non-natural amino acid, and polyethyleneglycole.

[0054] The term label is well-known to the skilled artisan and can be any desired structure of interest. Preferably such label can be selected from any known detectable groups, such as dyes, luminescent labeling groups such as chemiluminescent groups e.g. acridinium esters or dioxetanes, or fluorescent dyes e.g. fluorescein, coumarin, rhodamine, oxazine, resorufin, cyanine and derivatives thereof. Other examples of labeling groups are luminescent metal complexes such as ruthenium or europium complexes, enzymes as used for CEDIA (Cloned Enzyme Donor Immunoassay, e.g. EP-A-0 061 888), and radioisotopes.

[0055] Another preferred group of labels of interest for example comprises one partner of a bioaffine binding pair. While performing an assay this kind of label interacts specifically and preferably non-covalent with the other partner of the bioaffine binding pair. Examples of suitable binding partners of bioaffine binding pairs are hapten or antigen/antibody, biotin or biotin analogues such as aminobiotin, iminobiotin or destheiobiotin/avidin or streptavidin, sugar/lectin, nucleic acid or nucleic acid analogue/complementary nucleic acid, receptor/ligand e.g. steroid hormone receptor/steroid hormone. Preferred labels within this group are selected from hapten, antigen and hormone. Especially preferred labels are haptens like digoxin and biotin and analogues thereof.

[0056] Another group of preferred modifications are non-natural amino acids and derivatives thereof. Most interesting are non-natural amino acids containing functional groups, which are orthogonal to the natural amino acids, e.g. aldehyde functions, hydrazines, hydrazides, azids, and α-halogen-ketones.

[0057] In case the nucleophile comprises polyethyleneglycole (PEG), this PEG preferably has a molecular weight in the range of 2.000 Da to 50.000 Da. The PEG may be linear or branched. More preferred the PEG will be in the molecular weight range from 10.000 Da to 40.000 Da. Preferably the nucleophilic group of such nucleophile comprising PEG will be an arginine or a lysine having a free N-terminal α-amino group. This arginine or this lysine also may be the N-terminus of a peptide. Preferably such pegylated nucleophile is selected from the group consisting of Arg-His-PEG, Arg-His-Ala-PEG, Lys-His-PEG, Lys-His-Ala-PEG and Arg-His-Xaa-PEG, wherein Xaa may be any natural or non-natural di-amino carboxylic acid. The PEG-modified di-amino carboxylic acid may comprise one or two PEG molecule(s) bound to one or to both of these amino groups, respectively. The skilled artisan may select or design other appropriate PEG-modified nucleophiles, like a pegylated cysteine and others.

[0058] According to procedures known in the state of the art or according to the procedures given in the examples section and armed with the teaching of the present invention, it is now possible to obtain polynucleotide sequences coding for the trypsin mutants of the invention. Preferably the mutated trypsin according to the present invention is expressed as an inactive precursor (Zymogen) which is enzymatically cleaved to result in the active enzyme. In a further embodiment the present invention relates to a nucleotide sequence coding for the mutated trypsin comprising an amino acid substitution at position both at K60 and D189, and at least one more amino acid substitution by histidine at position N143 or position E151, respectively.

[0059] The present invention further includes an expression vector comprising a nucleic acid sequence according to the present invention operably linked to a promoter sequence capable of directing its expression in a host cell.

[0060] The present invention further includes an expression vector comprising a nucleic acid sequence according to the present invention operably linked to a promoter sequence capable of directing its expression in a host cell. Preferred vectors are plasmids such as pST and pYT shown in Figure 1.

[0061] Expression vectors useful in the present invention typically contain an origin of replication, a promoter located

upstream in the DNA sequence, and are followed by the DNA sequence coding for a trypsin mutant, followed by transcription termination sequences and the remaining vector. The expression vectors may also include other DNA sequences known in the art, for example, stability leader sequences which provide for stability of the expression product, secretory leader sequences which provide for secretion of the expression product, sequences which allow expression of the structural gene to be modulated (e.g., by the presence or absence of nutrients or other inducers in the growth medium), marking sequences which are capable of providing phenotypic selection in transformed host cells, and the sequences which provide sites for cleavage by restriction endonucleases.

[0062] The characteristics of the actual expression vector used must be compatible with the host cell, which is to be employed. For example, when cloning in an *E.*coli cell system, the expression vector should contain promoters isolated from the genome of *E.coli* cells (e.g., *lac,* or *trp).* Suitable origins of replication in *E.coli* various hosts include, for example, a ColE1 plasmid replication origin. Suitable promoters include, for example, *lac* and trp. It is also preferred that the expression vector includes a sequence coding for a selectable marker. The selectable marker is preferably an antibiotic resistance gene. As selectable markers, ampicillin resistance, or canamycin resistance may be conveniently employed. All of these materials are known in the art and are commercially available.

[0063] Suitable expression vectors containing the desired coding and control sequences may be constructed using standard recombinant DNA techniques known in the art, many of which are described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual (1989).

[0064] The present invention additionally concerns host cells containing an expression vector which comprises a DNA sequence coding for the mutant trypsin according to the present invention. Preferred are the host cells containing an expression vector comprising one or more regulatory DNA sequences capable of directing the replication and/or the expression of, and operatively linked to a DNA sequence coding for, all or a functional part of mutant trypsin. Suitable host cells include, for example, *E.coli* HB101 (ATCC 33694) available from Promega (2800 Woods Hollow Road, Madison, W1, USA), XL1-Blue MRF available from Stratagene (11011 North Torrey Pine Road, La Jolla, CA, USA) and the like.

[0065] Expression vectors may be introduced into host cells by various methods known in the art. For example, transformation of host cells with expression vectors can be carried out by polyethylene glycol mediated protoplast transformation method (Sambrook et al. 1989). However, other methods for introducing expression vectors into host cells, for example, electroporation, bolistic injection, or protoplast fusion, can also be employed.

[0066] Once an expression vector containing trypsin mutant has been introduced into an appropriate host cell, the host cell may be cultured under conditions permitting expression of the desired trypsin mutants. Host cells containing an expression vector which contains a DNA sequence coding for the trypsin mutant are, e.g., identified by one or more of the following general approaches: DNA hybridization, the presence or absence of marker gene functions, assessment of the level of transcription as measured by the production of trypsin mRNA transcripts in the host cell, and detection of the gene product immunologically.

[0067] It should, of course, be understood that not all expression vectors and DNA regulatory sequences would function equally well to express the DNA sequences of the present invention. Neither will all host cells function equally well with the same expression system. However, one of ordinary skill in the art will make a selection among expression vectors, DNA regulatory sequences, and host cells using the guidance provided herein without undue experimentation.

[0068] The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

[0069]

Figure 1 **Cloning vectors used for expression of mutant trypsinogen**
On the left a schematic for the E. *coli* shuttle vector pST, comprising a coding region for trypsinogen is shown. The coding region can be easily inserted into a pYT-vector, optimized for polypeptide expression in yeast. A schematic of a pYT-vector comprising a coding region for trypsinogen is given on the right hand of this figure.

Figure 2 **Kinetics of transamidation**
Time course of the transamidation of Ala-Ala-Tyr-Arg-His-Ala-Gly (triangles) with Arg-NH2 catalyzed by the trypsin variant Tn K60E, E151H, N143H, D189K resulting in Ala-Ala-Tyr-Arg-NH2 (circles) in the presence of a) 100 μM EDTA or b) 100 μM ZnCl2. Squares represent AAY, which is formed as a side product of reaction due to proteolysis.

Figure 3 **Influence of Zn^{2+} on catalytic activity**

The influence of the presence (grey bars) or absence (black bars) of Zn^{2+} ions on the rate of peptide turnover by trypsin variant Tn K60E, E151H, N143H, D189K is shown.

Figure 4 Influence of the recognition sequence on the rate of reaction catalyzed by the trypsin variant Tn K60E, E151H, N143H, D189K-catalyzed.
Variant trypsin according to the present invention has been tested for catalytic activity upon peptide substrates with different peptide sequences at or close to the cleavage site. The initial rate (v) of peptide consumption is given in nM/min

Figure 5 Mass spectrum of Bz-Ala-Ala-Tyr-Arg-His-Lys (6-CF)-OH

Examples

Example 1

General procedure of generating trypsin variants:

[0070]

1. Introduction of the desired mutations into trypsin or trypsinogen using a suitable vector comprising the DNA encoding for trypsin or trypsinogen, e.g., a pST vector (cf. Fig.: 1). The *E. coli* vector pST has been originally constructed from L. Hedstrom and represents a yeast shuttle vector containing an ADH/GAPDH-promoter and α-factor leader sequence fused to a sequence encoding for trypsinogen; see: Hedstrom, L., et al., Science 255 (1992) 1249-1253.

2. Transformation of the constructed vector e.g. in E. *coli.*

3. Sub cloning of the modified trypsin-and trypsinogen sequence, respectively, using suitable expression vectors, e.g. yeast vector pYT (cf. Fig.: 1). In the case the desired mutation was introduced in this expression vector directly, this step is not needed.

4. Expression of the modified trypsin or trypsinogen in *E. coli* or yeast.

5. Isolation of the modified trypsin or trypsinogen using suitable separation methods, e.g. cation exchange chromatography.

6. In the case trypsinogen has been expressed, the isolated zymogene needs to be activated by limited proteolysis with enterokinase.

7. Final purification of the activated trypsin applying suitable purification methods, e.g. affinity chromatography or anion exchange chromatography.

8. Dialysis

[0071] In Table 1 the primary sequence of the trypsin variant Tn K60E, E151H, N143H, D189K (corresponding to mutated anionic rat trypsin II, without signal sequence and pro-sequence) is given.

Table 1:

Basic characteristics of the trypsin variant K60E, N143H, E151H, D183K		
mass	position	peptide sequence (= SEQ ID NO.: 1)
23828.5978	1-223	IVGGYTCQENSVPYQVSLNS GYHFCGGSLINDQWWSAAH CYESRIQVRLGEHNINVLEG NEQFVNAAKIIKHPNFDRKT LNNDIMLIKLSSPVKLNARV ATVALPSSCAPAGTQCLISG

(continued)

Basic characteristics of the trypsin variant K60E, N143H, E151H, D183K		
mass	position	peptide sequence (= SEQ ID NO.: 1)
		WGHTLSSGVNHPDLLQCLDA PLLPQADCEASYPGKITDNM VCVGFLEGGKKSCQGDSGGP WCNGELQGIVSWGYGCALP DNPGVYTKVCNYVDWIQDTI AAN

(Mutations are in bold)

Theoretical peptide mass:

[Theoretical pI: 5.41 / Mw (average mass): 23843.00 / Mw (monoisotopic mass): 23827.59]

Example 2

Preparation of the peptides by means of solid phase peptide synthesis

[0072] Unless specified otherwise the peptides mentioned in this application were synthesized by means of fluorenyl-methyloxycarbonyl-(Fmoc)-solid phase peptide synthesis on a batch peptide synthesizer e.g. from Applied Biosystems A433. In each case 4.0 equivalents of the amino acid derivative shown in Table 2 were used for this process.

Table 2:

A	Fmoc-Ala-OH
C	Fmoc-Cys(Trt)-OH
D	Fmoc-Asp(OtBu)-OH
E	Fmoc-Glu(OtBu)-OH
F	Fmoc-Phe-OH
G	Fmoc-Gly-OH
H	Fmoc-His(Trt)-OH
I	Fmoc-Ile-OH
K	Fmoc-Lys(Boc)-OH
L	Fmoc-Leu-OH
M	Fmoc-Met-OH
N	Fmoc-Asn(Trt)-OH
P	Fmoc-Pro-OH
Q	Fmoc-Gln(Trt)-OH
R	Fmoc-Arg(Pbf)-OH
S	Fmoc-Ser(tBu)-OH
T	Fmoc-Thr(tBu)-OH
V	Fmoc-Val-OH
W	Fmoc-Trp-OH
Y	Fmoc-Tyr(tBu)-OH

[0073] The amino acid derivatives were dissolved in N-methyl-2-pyrrolidinon. The peptide was synthesized on Wang

resin (Wang, S.-S., J. Am. Chem. Soc. 95 (1973) 1328-1333) or on 2-chlortrityl chloride-resin (Barlos, K., et al., Tetrahedron Lett. 30 (1989) 3947-3950). The resin was loaded with 0.5 to 1.0 mMol/g. The coupling reactions were carried out for 20 minutes using 4 equivalents dicyclohexylcarbodiimide and 4 equivalents N-hydroxybenzotriazole in dimethylformamide relative to the Fmoc-amino acid derivative in dimethylformamide as the reaction medium. The Fmoc group was cleaved after each step of the synthesis with 20 % piperidine in dimethylformamide for 20 min. Terminal amino groups on the solid phase were optionally acetylated with acetic anhydride.

[0074] The introduction of a label e.g. a metal chelate label or a fluorescein label or of a PEG at the C-terminus was carried out during the solid phase synthesis by the direct incorporation of for example a metal chelate or fluorescein coupled amino acid derivative (described in WO 96/03409).

[0075] The peptide was released from the support and the acid-labile protective groups were cleaved with 20 ml trifluoroacetic acid, 0.5 ml ethanediol, 1 ml thioanisole, 1.5 g phenol and 1 ml water within 40 min at room temperature. Depending on the amino acid derivatives that were used, it is also possible to use cocktails containing fewer radical traps. 300 ml cooled diisopropyl ether was subsequently added to the reaction solution and was kept for 40 min at 0° C in order to completely precipitate the peptide. The precipitate was filtered, washed with diisopropyl ether and dissolved in a small amount of 50 % acetic acid and lyophilized. The crude material obtained was purified by means of preparative HPLC on Vydac RP C18 218TP152050 (column 50 x 250 mm, 300 Å; 15 μm) over an appropriate gradient (eluant A: water, 0.1% trifluoroacetic acid, eluant B: acetonitrile, 0.1 % trifluoroacetic acid) within ca. 120 min. The eluted material was identified by mass spectrometry.

[0076] Alternatively the label, e.g. PEG can also be introduced after cleavage of the peptide from the resin. For this it was advantageous to use a chlortrityl chloride-resin. The protected peptide was cleaved off the resin with 1 % trifluoroacetic acid in 10 ml dichloromethane for 20 min at room temperature. Then the C-terminus of the peptide was activated by 2 equivalents dicyclohexylcarbodiimide, 2 equivalents N-hydroxybenzotriazole and 2 equivalents triethylamine in dimethylformamide as reaction medium and one equivalent of the amino acid derivative of the labeling group or the effector group was added. The protective groups are removed by using 20 ml trifluoroacetic acid, 0.5 ml ethanediol, 1 ml thioanisole, 1.5 g phenol and 1 ml water within 40 min at room temperature. Depending on the amino acid derivatives that were used, it is also possible to use cocktails containing fewer radical traps. 300 ml cooled diisopropyl ether was subsequently added to the reaction solution and the reaction solution was kept for 40 min at 0 °C in order to completely precipitate the peptide. The HPLC purification was carried out as described above.

Example 3

Transamidation of Ala-Ala-Tyr-Arg-His-Ala-Gly with Arg-NH_2 catalyzed by the trypsin variant Tn K60E, E151H, N143H, D189K.

[0077] The peptide Ala-Ala-Tyr-Arg-His-Ala-Gly was synthesized by conventional solid-phase peptide synthesis using Fmoc-chemistry and a preloaded Wang-resin as described in Example 2. The respective amino acid building blocks are commercially available and were purchased from various suppliers. Arg-NH_2 was a commercial product from Bachem (Switzerland).

[0078] 1 ml reaction volume containing 1 mM Ala-Ala-Tyr-Arg-His-Ala-Gly (SEQ ID NO: 2) and 5 mM Arg-NH_2 dissolved in 0.1 M Hepes buffer pH 8.0; 20 μM trypsin variant Tn K60E, E151H, N143H, D189K; 100 μM $ZnCl_2$ or, alternatively 100 μM EDTA was stirred at 25 °C. After defined time intervals aliquots were withdrawn and reaction quenched by addition of 1% trifluoroacetic acid in methanol/water (1:1, v/v) resulting in a final pH of 2 of the withdrawn samples. The latter were analyzed by analytical HPLC giving the time courses of the reactions as shown in Fig. 2. The identity of the final product of synthesis was verified by mass spectroscopy.

Example 4

Influence of Zn^{2+} ions on the specificity of the trypsin variant Tn K60E, E151H, N143H, D189K.

[0079] The peptide substrates Bz-Ala-Ala-Tyr-Arg-His-Ala-Ala-Gly, Bz-Ala-Ala-Tyr-Arg-His-Ala-Gly, Bz-Ala-Ala-Tyr-Arg-His-Asp-Ala-Gly, Bz-Ala-Ala-Tyr-Arg-Arg-Ala-Gly, and Bz-Ala-Ala-Tyr-Asp-His-Ala-Gly was synthesized by conventional solid-phase peptide synthesis using Fmoc-chemistry and a preloaded Wang-resin. The respective amino acid building blocks are commercially available and were purchased from various suppliers.

[0080] 1 ml reaction volume containing 1 mM of one of the following peptides Bz-Ala-Ala-Tyr-Arg-His-Ala-Ala-Gly (SEQ ID NO: 3), Bz-Ala-Ala-Tyr-Arg-His-Ala-Gly (SEQ ID NO: 4), Bz-Ala-Ala-Tyr-Arg-His-Asp-Ala-Gly (SEQ ID NO: 5), Bz-Ala-Ala-Tyr-Arg-Arg-Ala-Gly (SEQ ID NO: 6) or Bz-Ala-Ala-Tyr-Asp-His-Ala-Gly (SEQ ID NO: 7) dissolved in 0.1 M Pipes/Tris-buffer pH 8.0; 20 μM trypsin variant Tn K60E, E151H, N143H, D189K, 100 μM $ZnCl_2$ or, alternatively EDTA, was stirred at 30 °C. After defined time intervals, the reactions were terminated by addition of 1 % trifluoroacetic acid in

methanol/water (1:1, v/v). The quenched reaction mixtures were analyzed by analytical HPLC. The respective rates of reactions are shown in Fig. 3. The identity of the final products was verified by mass spectroscopy.

Example 5

Influence of the recognition sequence on the rate of reaction catalyzed by the trypsin variant Tn K60E, E151H, N143H, D189K.

[0081] The N^{α}-benzoylated peptides of SEQ ID NOs: 3-19 have been synthesized by conventional solid-phase peptide synthesis using Fmoc-chemistry and a preloaded Wang-resin. The respective amino acid building blocks are commercially available and were purchased from various suppliers.

[0082] 1 ml reaction volume containing 1 mM N^{α}-benzoylated peptide dissolved in 0.1 M Pipes/Tris-buffer pH 8.0; 20 μM trypsin variant Tn K60E, E151 H, N143H, D189K; 100 μM $ZnCl_2$ was stirred at 30 °C. After defined time intervals, the reactions were terminated by adding of 1 % trifluoroacetic acid in methanol/water (1:1, v/v).

[0083] The quenched reaction mixtures were analyzed by analytical HPLC. The respective rates of reactions are shown in Fig. 4. The identity of the final products was verified by mass spectroscopy.

[0084] As can be easily seen from Fig. 4 the trypsin variant Tn (=K60E, E151H, N143H, and D189K) has a strong preference for a cleavage site comprising L, F or Y in position P_1, R or K in position P_1' and His in position P_2'.

Example 6

Transamidation of Bz-Ala-Ala-Tyr-Arg-His-Ala-Gly with Arg-His-Ala-Lys(6-CF)-OH catalyzed by the trypsin variant Tn K60E, E151H, N143H, D189K.

[0085] Bz-Ala-Ala-Tyr-Arg-His-Ala-Gly has been synthesized by conventional solid-phase peptide synthesis using Fmoc-chemistry and a preloaded Wang-resin. The respective amino acid building blocks are commercially available and were purchased from various suppliers. Arg-His-Ala-Lys(6-CF)-OH was synthesized by fragment condensation. The protected tripeptide Boc-Arg$(Boc)_2$-His(Trt)-Ala-OH was synthesized on a chlorotrityl-resin using conventional solid phase peptide synthesis. The peptide was cleaved from the resin with 2 x 40 ml of a cocktail containing methylene chloride/acetic acid/trifluoro acetic acid (v/v/v 8/1/1). The crude material was purified by reversed phase HPLC. The synthesis of the other fragment, Fmoc-Lys(6-carboxy-fluorescein), was performed from 0.6 mmol Fmoc-Lys*HCl, 0,655 mmol 6-carboxyfluorescein (purchased from Molecular Probes) in 3 ml dioxin and 3 ml DMF. The Fmoc-group was cleaved off with piperidine and the crude material purified by reversed phase HPLC. Then the protected tripeptide was activated with 1 equivalent of HBTU (Iris Biotech) and 3 equivalents of diisopropylethylamine in DMF. 1 equivalent of Lys(6-carboxy-fluorescein) was added and the mixture was stirred for 2 h at room temperature. Then the deprotection was done using a deprotection cocktail (18 ml trifluoroacetic acid, 0.5 ml water and 0.5 ml ethandithiol). The peptide was precipitated with diisopropylether, purified by RP-HPLC, and obtained in good yield.

[0086] 1 ml reaction volume containing 0.5 mM Bz-Ala-Ala-Tyr-Arg-His-Ala-Gly (SEQ ID NO: 20) and 2.5 mM Arg-His-Ala-Lys(6-CF)-OH dissolved in 0.1 M Pipes/Tris-buffer pH 8.0; 20 μM trypsin variant Tn K60E, E151H, N143H, D189K; 100 μM $ZnCl_2$ or, alternatively 100 μM EDTA, was stirred at 30°C. After defined time intervals respective aliquots were withdrawn and quenched by addition of 1 % trifluoroacetic acid in methanol/water (1:1, v/v) resulting in a final pH of 2 of the withdrawn samples. HPLC was used for analyzing the course of reaction and isolating the synthesis product. The latter was obtained in a yield of >99% and has been further analyzed by mass spectroscopy as shown in Fig. 5.

Example 7

[0087] Transamidation of AKTAAALHIL VKEEKLALDL LEQIKNGADF GKLAKKHSIC PSGKRGGDLG EFRQGQMVPA FDKWFSCPV LEPTGPLHTQ FGYHIIKVLY RH with Arg-His-Gly-PEG catalyzed by the trypsin variant Tn K60E, E151H, N143H, D189K.

[0088] Arg-His-Gly-PEG is synthesized by fragment condensation of Boc-Arg$(Boc)_2$-His(Trt)-Gly-OH and amino-PEG (20kD) purchased form Nektar/Shearwater. For synthesis of the protected tripeptide and the activation of the fragment see example 6. Here 0.5 equivalents of amino-PEG (20 kD) were used as nucleophile. After deprotection (cocktail see example 6) all low molecular impurities were separated off using RP-HPLC.

[0089] The polypeptide AKTAAALHIL VKEEKLALDL LEQIKNGADF GKLAKKHSIC PSGKRGGDLG EFRQGQMVPA FDKWFSCPV LEPTGPLHTQ FGYHIIKVLY RH (SEQ ID NO: 21), containing the recognition sequence Tyr-Arg-His on its C-terminus, is produced from native E. *coli* parvulin 10 by exchanging the original C-terminal Asn moiety with an artificial His. After expression and purification, the respective modified parvulin 10 (Asn92His) variant is dissolved in Pipes/Tris-buffer pH 8.0. Final concentrations of 20 μM trypsin variant Tn K60E, E151H, N 143H, D189K, 100 μM $ZnCl_2$,

and an excess of Arg-His-Gly-PEG are added into this reaction mixture. After stirring at 30 °C the reaction is terminated by addition of 1 % trifluoroacetic acid in methanol/water (1:1, v/v). Analysis is done by HPLC, gel electrophoresis and/or mass spectroscopy. Isolation of the final product AKTAAALHIL VKEEKLALDL LEQIKNGADF GKLAKKHSIC PSGKRG-GDLG EFRQGQMVPA FDKWFSCPV LEPTGPLHTQ FGYHIIKVLY RH-Gly-PEG is performed by conventional protein purification techniques, e.g. by chromatographic methods.

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SEQUENCE LISTING

[0091]

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F. Hoffmann-La Roche GmbH

<120> C-terminal modification of polypeptides

<130> 22684 WO

<150> EP 04019237.9
<151> 2004-08-13

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```

Claims

1. A mutated trypsin comprising an amino acid substitution both at position K60 and D189, and at least one more amino acid substitution by histidine at position N143 or position E151.

2. The mutated trypsin of claim 1, wherein K60 is substituted by E or D.

3. The mutated trypsin of claim 1, wherein D 189 is substituted by K, H or R.

4. A method of producing a C-terminally transacylated target peptide comprising the steps of:

   (a) providing a polypeptide comprising a target peptide and a restriction site peptide comprising the cleavage site Xaa_1-Xaa_2-His, wherein Xaa_1 is L, Y or F, and Xaa_2 is R or K, wherein said restriction site peptide overlaps with the target peptide by the amino acid Xaa_1 at the C-terminal end of said target peptide,
   (b) bringing said peptide into contact with a trypsin mutant according to any of claims 1 to 3 under conditions allowing for endoproteolytic cleavage after Xaa_1 and formation of an endoprotease target peptide peptide-acyl-intermediate,
   (c) adding an appropriate nucleophile and
   (d) upon nucleophilic attack and binding of said nucleophile to the C-terminus of the target peptide releasing the mutated trypsin from the endoprotease target peptide-acyl-intermediate.

5. The method of claim 4, wherein said nucleophile is selected from the group consisting of primary amines, imines, secondary amines, thiol and hydroxyl.

6. The method of claim 4, wherein said nucleophile comprising modification is selected from the group consisting of an amino acid amide a peptide, a peptide amide, a label, a labeled amino acid amide, a labeled peptide, a labeled peptide amide, and polyethyleneglycole.

7. The method of claim 6, wherein said modification is polyethyleneglycole,

8. A nucleotide sequence coding for the mutated trypsin as defined in any of claims 1 to 3.

Patentansprüche

1. Mutiertes Trypsin, umfassend eine Aminosäuresubstitution sowohl in Position K60 als auch D189 sowie wenigstens eine weitere Aminosäuresubstitution durch Histidin in Position N143 oder Position E151.

2. Mutiertes Trypsin nach Anspruch 1, wobei K60 durch E oder D substituiert ist.

3. Mutiertes Trypsin nach Anspruch 1, wobei D189 durch K, H oder R substituiert ist.

4. Verfahren zur Herstellung eines C-terminal transacylierten Zielpeptids, wobei man in den Verfahrensschritten

   (a) ein Polypeptid, umfassend ein Zielpeptid und ein Restriktionsstellenpeptid, umfassend die Spaltstelle Xaa_1-Xaa_2-His, wobei Xaa_1 für L, Y oder F und Xaa_2 für R oder K steht, bereitstellt, wobei das Restriktionsstellenpeptid mit dem Zielpeptid um die Aminosäure Xaa_1 am C-terminalen Ende des Zielpeptids überlappt,
   (b) das Peptid mit einer Trypsinmutante gemäß einem der Ansprüche 1 bis 3 unter Bedingungen in Kontakt bringt, die die endoproteolytische Spaltung hinter Xaa_1 und die Bildung einer Endoprotease-Zielpeptid-Peptidacyl-Zwischenstufe gestatten,
   (c) ein geeignetes Nukleophil zugibt und
   (d) nach nukleophilem Angriff und Bindung des Nukleophils an den C-Terminus des Zielpeptids das mutierte Trypsin aus der Endoprotease-Zielpeptid-Peptidacyl-Zwischenstufe freigesetzt wird.

5. Verfahren nach Anspruch 4, wobei das Nukleophil aus der Gruppe primäre Amine, Imine, sekundäre Amine, Thiol und Hydroxyl ausgewählt ist.

6. Verfahren nach Anspruch 4, wobei die das Nukleophil umfassende Modifikation aus der Gruppe Aminosäureamid, Peptid, Peptidamid, Markierung, markiertes Aminosäureamid, markiertes Peptid, markiertes Peptidamid und Polyethylenglykol ausgewählt ist.

7. Verfahren nach Anspruch 6, wobei es sich bei der Modifikation um Polyethylenglykol handelt.

8. Nukleotidsequenz, codierend für das mutierte Trypsin mit der in einem der Ansprüche 1 bis 3 angegebenen Bedeutung.

Revendications

1. Trypsine mutée comprenant une substitution d'acide aminé à la fois en position K60 et D189, et au moins une autre substitution d'acide aminé par de l'histidine en position N143 ou en position E151.

2. Trypsine mutée selon la revendication 1, dans laquelle K60 est remplacé par E ou D.

3. Trypsine mutée selon la revendication 1, dans laquelle D189 est remplacé par K, H ou R.

4. Procédé de production d'un peptide cible transacylé à l'extrémité C comprenant les étapes de :

   (a) fourniture d'un polypeptide comprenant un peptide cible et un peptide à site de restriction comprenant le site de clivage Xaa_1-Xaa_2-His, dans lequel Xaa_1 représente L, Y ou F, et Xaa_2 représente R ou K, dans lequel ledit peptide à site de restriction chevauche le peptide cible par l'acide aminé Xaa_1 au niveau de l'extrémité C terminale dudit peptide cible,
   (b) porter ledit peptide au contact d'un mutant de la trypsine selon l'une quelconque des revendications 1 à 3 dans des conditions permettant un clivage endoprotéolytique après Xaa_1 et la formation d'un intermédiaire peptide cible d'endoprotéase peptide-acyle,
   (c) ajout d'un nucléophile approprié et
   (d) dès l'attaque nucléophile et la liaison dudit nucléophile à l'extrémité C du peptide cible, libération de la trypsine mutée de l'intermédiaire peptide cible d'endoprotéase-acyle.

5. Procédé selon la revendication 4, dans lequel ledit nucléophile est choisi dans le groupe constitué par les amines primaires, les imines, les amines secondaires, le thiol et l'hydroxyle.

6. Procédé selon la revendication 4, dans lequel ledit nucléophile comprenant une modification est choisi dans le groupe constitué par un amide d'acide aminé, un peptide, un amide peptidique, une marqueur, un amide d'acide aminé marqué, un peptide marqué, un amide peptidique marqué, et le polyéthylène glycol.

7. Procédé selon la revendication 6, dans lequel ladite modification est le polyéthylène glycol.

8. Séquence nucléotidique codant pour la trypsine mutée selon l'une quelconque des revendications 1 à 3.

Fig. 1

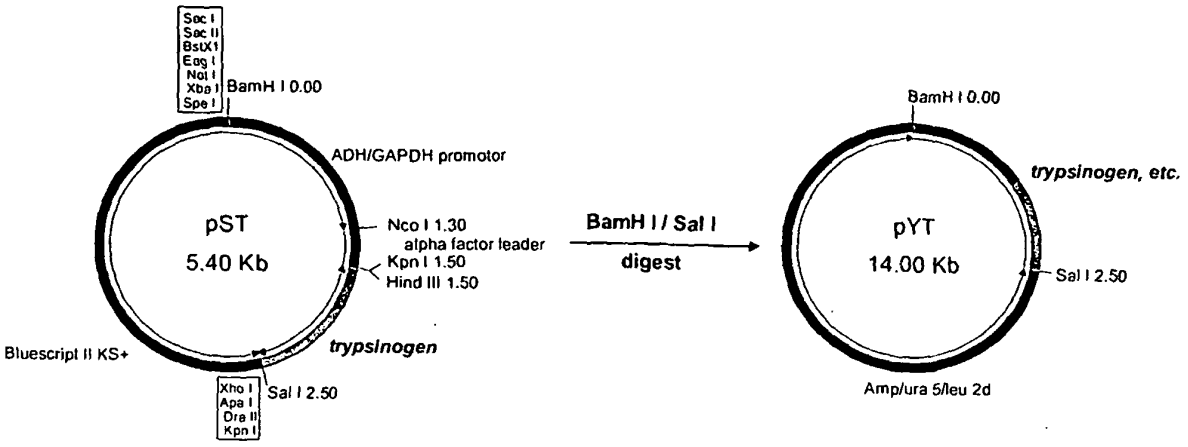

Sac I
Sac II
BstX1
Eag I
Not I
Xba I
Spe I
BamH I 0.00
ADH/GAPDH promotor
pST
5.40 Kb
Nco I 1.30
alpha factor leader
Kpn I 1.50
Hind III 1.50
Bluescript II KS+
trypsinogen
Xho I
Apa I
Ora II
Kpn I
Sal I 2.50
BamH I / Sal I
digest
BamH I 0.00
trypsinogen, etc.
pYT
14.00 Kb
Sal I 2.50
Amp/ura 5/leu 2d

Fig. 2

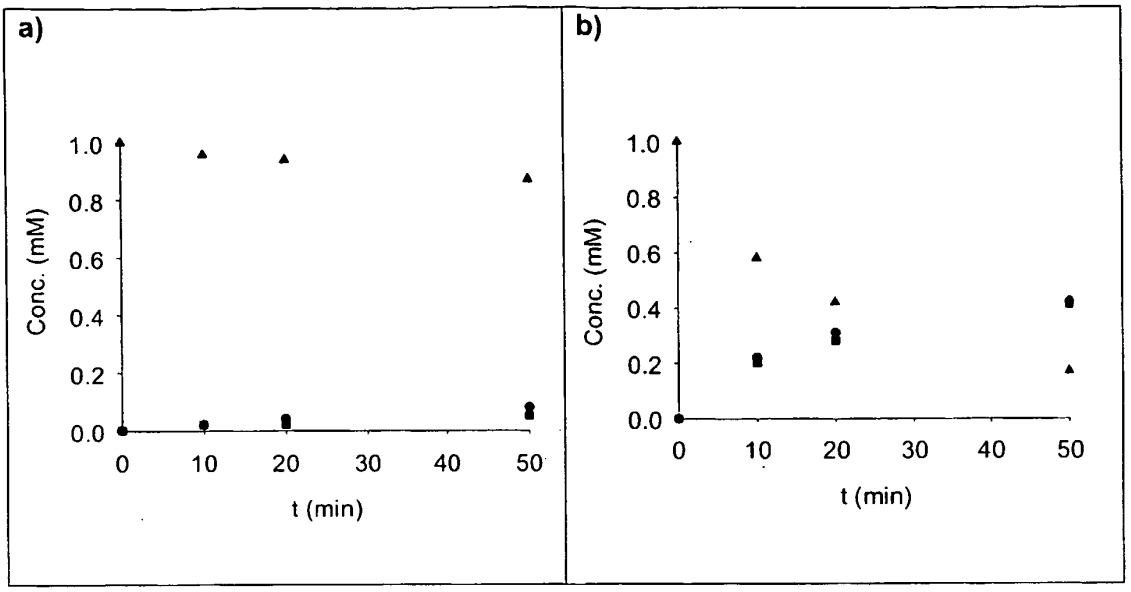

a)
1.0
0.8
0.6
0.4
0.2
0.0
Conc. (mM)
0
10
20
30
40
50
t (min)
b)
1.0
0.8
0.6
0.4
0.2
0.0
Conc. (mM)
0
10
20
30
40
50
t (min)

Fig. 3

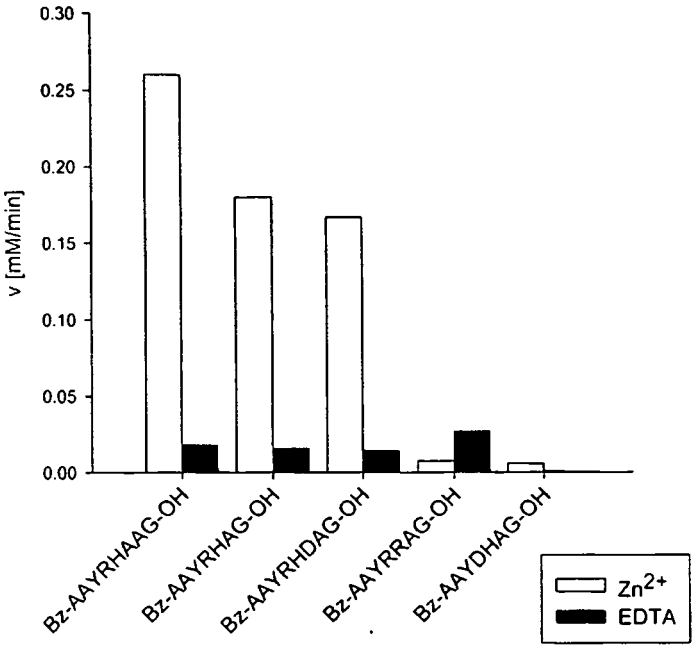

0.30
0.25
0.20
0.15
0.10
0.05
0.00
v [mM/min]
Bz-AAYRHAAG-OH
Bz-AAYRHAG-OH
Bz-AAYRHDAG-OH
Bz-AAYRRAG-OH
Bz-AAYDHAG-OH
Zn2+
EDTA

Fig. 4

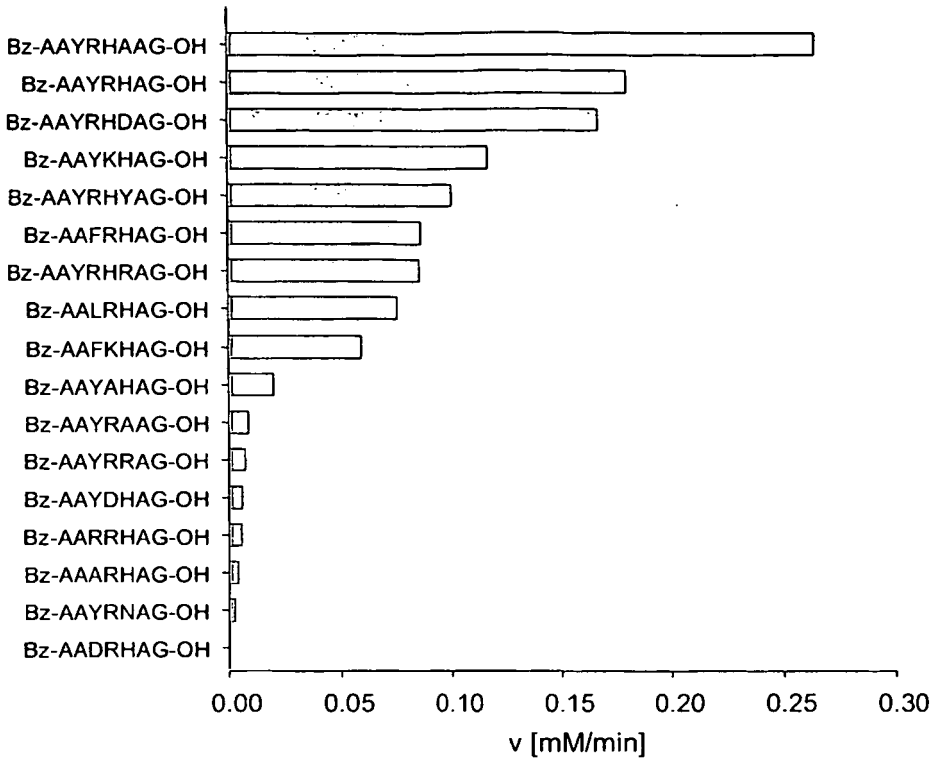
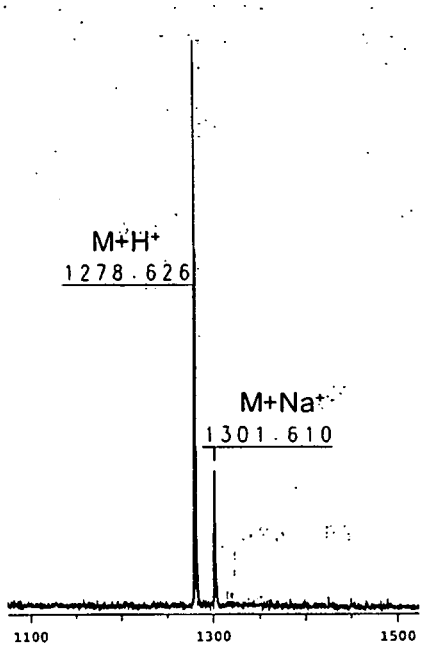

Bz-AAYRHAAG-OH
Bz-AAYRHAG-OH
Bz-AAYRHDAG-OH
Bz-AAYKHAG-OH
Bz-AAYRHYAG-OH
Bz-AAFRHAG-OH
Bz-AAYRHRAG-OH
Bz-AALRHAG-OH
Bz-AAFKHAG-OH
Bz-AAYAHAG-OH
Bz-AAYRAAG-OH
Bz-AAYRRAG-OH
Bz-AAYDHAG-OH
Bz-AARRHAG-OH
Bz-AAARHAG-OH
Bz-AAYRNAG-OH
Bz-AADRHAG-OH
0.00
0.05
0.10
0.15
0.20
0.25
0.30
v [mM/min]

Fig. 5



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